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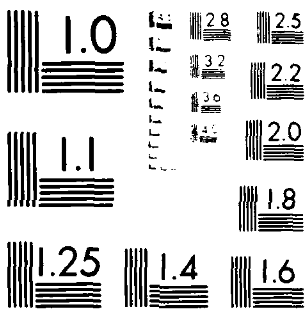


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# NONDESTRUCTIVE BIOPHYSICAL PROBES OF THE BASIS AND MECHANISM OF RESISTANCE IN MICROBIAL SPORES


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## ABSTRACT

Bacterial spores possess extraordinary resistance against destruction by heat and other deleterious agents, so compensatory safeguards must be taken in medicine and industry. Spores furthermore exemplify the general biological phenomenon of dormancy. The basis and mechanism accounting for these remarkable properties remain largely unknown despite a century of research. The project is aimed at solving this puzzle with the rationale of using biophysical probes that do not destroy the cellular and molecular configurations conferring resistance in intact spores. The project involves active collaboration by investigators at two universities and continuing cooperation within an international interdisciplinary program on spore resistance mechanisms.

In a 1980 paper, a theory was proposed accounting for spore heat resistance based on partial dehydration of the core protoplast through a mechanism of reverse osmosis exerted by pressure from anisotropic cortex growth. In a 1981 paper, the preparation of homogeneous salt forms of spores by ion exchange was described; the order of resistance among the specifically mineralized spores was MN> native> Ca or Mg> K> Na or H. Also in 1981, a special 87-page issue of Spore Newsletter contained eight 2-page articles by project personnel, which were presented at an international interdisciplinary seminar-workshop on spore resistance mechanisms coconvened by the project principal investigator. A 1982 paper described the sensitivity of various salt forms of spores to the germinating action of hydrostatic pressure. In a series of three 1982 papers: (1) the heat resistance of spores was correlated with their water content, wet density, and protoplast/sporoplast volume ratio; (2) photometric immersion refractometry was used to show that the total water content is distributed unequally within the dormant spore, and that the sporoplast becomes more refractile and therefore more dehydrated as the heat resistance becomes greater among various types of spores; (3) wet and dry densities of various spores determined by buoyant sedimentation with various media were compared with the values obtained by conventional mass measurement, the difference being accountable from the permeation of media of low molecular weight into the spores. In 1983, dielectric characterization showed that isolated forespores have unusually low conductivity and suggested that forespore dehydration may result from exit of ions into the sporangium. A 1983 review focused on the nature of dehydration during sporogenesis, the physical state of spore electrolytes, and the relationship of heat resistance to mineralization in spores. Another 1983 review focused on electrochemical and mechanical interactions in the mureins of the vegetative cell wall and the spore cortex.

## STATEMENT OF THE PROBLEM STUDIED

Bacterial spores possess extraordinary resistance against destruction by heat and other deleterious agents, so compensatory safeguards must be taken in medicine and industry. Spores furthermore exemplify the general biological phenomenon of dormancy. The basis and mechanism accounting for these remarkable properties remain largely unknown despite a century of research. This project is aimed at solving this puzzle with the rationale of using biophysical probes that do not destroy the cellular and molecular configurations conferring resistance in intact spores. For example, dielectric measurements were used to characterize the physicochemical states of small electrolytes and water within the spore, and photometric immersion refractometry was used to determine if dehydration of the protoplast accounts for sporal resistance to heat. These and other approaches employed a wide representation of bacterial spore types varying greatly in measured thermoresistance. The project involved active collaboration by investigators at two universities and continuing cooperation within an international interdisciplinary program on spore resistance mechanisms.

## PARTICIPATING SCIENTIFIC PERSONNEL

At Michigan State University: John Algie, Teofila C. Beaman, Thomas Corner, Philipp Gerhardt, Tomihiko Koshikawa, Stuart Pankratz, Louis Tisa.

At University of Rochester: Gary Bender, Edwin Carstensen, Sally Child, Robert Marquis

"D. Sc." awarded with honors to John Algie by the University of Toulouse, France.

## SUMMARIES OF RESULTS AND LIST OF PUBLICATIONS

Reproduced below are the reprint or preprint abstracts of published papers from USARO-supported research, together with the journal reference citations.

CURRENT MICROBIOLOGY, Vol. 3 (1980), pp. 287-290

**Current Microbiology**
An International Journal

# The Heat Resistance of Bacterial Spores due to Their Partial Dehydration by Reverse Osmosis

John E. Algie

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824, USA

**Abstract.** The ability of bacterial spores to withstand heat is known to be associated with a lowering of their water content. This partial dehydration is considered to be produced by reverse osmosis, with the pressure being applied by the cortex as it is growing. Experiments show that the cortex is capable of supplying the pressure.

Marquis, R.E., E.L. Carstensen, S.Z. Child, and G.R. Bender. 1981. Preparation and characterization of various salt forms of Bacillus megaterium spores, p. 266-268. In H.S. Levinson, A.L. Sonensheim, and D.J. Tipper (ed.), Sporulation and germination. American Soc. for Microbiol., Washington, D.C.

# Preparation and Characterization of Various Salt Forms of *Bacillus megaterium* Spores

R. E. MARQUIS, E. L. CARSTENSEN, S. Z. CHILD, AND G. R. BENDER

*Departments of Microbiology and Electrical Engineering, The University of Rochester, Rochester, New York 14642*

Spores of *Bacillus megaterium* ATCC 19213 were subjected to an ion-exchange regimen that resulted in nearly complete exchange of cations from the core and enveloping structures without major viability losses. The extreme immobilization of electrolytes characteristic of bacterial spores was found not to be related specifically to calcification. The state of hydration of the spores in aqueous media was not affected by conversion of one salt form to another, but heat resistance was. The order of resistance among the salt forms was Mn > native > Ca, Mg > K > Na, H.

*Gerhardt, P., and W.G. Murrell (ed.). 1981. Bases and mechanisms of bacterial spore resistance. Spore Newsletter 8(5):1-83. This special issue includes eight 2-page articles by project personnel, the citations for which are listed below. A copy of the complete Spore Newsletter is sent to USARO

a. Beaman, T.C., J.T. Greenamyre, T.R. Corner, H.S. Pankratz, and P. Gerhardt. 1981. Heat Resistance of Bacterial Spore Types Correlated with Wet Density, Water Content, and Inner:Outer Protoplast Volume Ratio. pp. 8-9. (in above).

b. Tisa, L.S., J.E. Algie and P. Gerhardt. 1981. Isopycnic Density Gradient Determination of Wet and Dry Densities of Representative Bacterial Spore Types. pp. 10-11. (in above).

c. Gerhardt, P., T.C. Beaman, T.R. Corner, J.T. Greenamayer and L.S. Tisa. 1981. Photometric Immersion Refractometry of Bacterial Spores. pp. 12-13. (in above).

d. Algie, J.E. and L. Tisa. 1981. The Effect of Water Activity of Core and Cortex on the Heat Resistance of Bacterial Spores. pp. 20-21. (in above).

e. Koshikawa, T., J.E. Algie, L.S. Tisa and P. Gerhardt. 1981. Effect of Water Activity on Heat Resistance of Vegetative Cells of Bacillus megaterium. pp. 24-25. (in above).

-- f. Marquis, Robert E., E.L. Carstensen, G.R. Bender, and S. Child. 1981. Ion Exchange, Dielectric Properties and Heat Resistance of Bacterial Spores. pp. 34-35. (in above).

g. Carstensen, E.L. and R.E. Marquis. 1981. Absorption of Ultrasound by Spores and Vegetative Cells. pp. 48-49. (in above).

h. Algie, J.E. 1981. Theories in Sporulation and Heat Resistance. pp. 72-74. (in above).

## Sensitivity of various salt forms of *Bacillus megaterium* spores to the germinating action of hydrostatic pressure

GARY R. BENDER AND ROBERT E. MARQUIS

*Department of Microbiology, School of Medicine and Dentistry, The University of Rochester, Rochester, NY, U.S.A. 14642*

Accepted February 18, 1982

BENDER, G. R., and R. E. MARQUIS. 1982. Sensitivity of various salt forms of *Bacillus megaterium* spores to the germinating action of hydrostatic pressure. Can. J. Microbiol. **28**: 643–649.

Spores of *Bacillus megaterium* ATCC 19213 were subjected to a complete ion-exchange regimen, which included titration to a pH value of 2, heating at 60°C for up to 18 h, back titration with various base solutions to a pH value of 8, and heating again at 60°C. Spore populations maintained high levels of viability throughout this rigorous procedure, and the various salt forms prepared showed a wide range of sensitivities to the germinating effect of hydrostatic pressure. Native spores showed the expected germination response when subjected to pressures of 350–750 atm (1 atm = 101.325 kPa) at 24°C. There was a threshold pressure for germination of some 300 atm, and the apparent activation volume for the process was calculated to be 188 mL/mol, indicating that these spores had approximately the same pressure sensitivity as those of *Bacillus subtilis* or *Bacillus pumilus*. The optimum pH for germination was approximately 8, and the optimum temperature was approximately 45°C. The hierarchy of resistance of the various salt forms tested to the germinating action of 493 atm pressure was H > K > Ca, Mg, Na > native. The H form was particularly insensitive, even to pressures as high as 1020 atm, but germinated in response to chemical germinants. We concluded that the specific mineralization of bacterial spores has major influence on pressure-induced germination, which can occur even in the absence of added salts. Sensitivities to pressure could not be correlated with previously reported heat sensitivities, electrostasis, or states of dehydration.

JOURNAL OF BACTERIOLOGY, May 1982, p. 870–877
0021-9193/82/050870-08$02.00/0

Vol. 150, No. 2

# Bacterial Spore Heat Resistance Correlated with Water Content, Wet Density, and Protoplast/Sporoplast Volume Ratio†

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Received 3 June 1981/Accepted 22 October 1981

Five types of dormant *Bacillus* spores, between and within species, were selected representing a 600-fold range in moist-heat resistance determined as a $D_{100}$ value. The wet and dry density and the solids and water content of the entire spore and isolated integument of each type were determined directly from gram masses of material, with correction for interstitial water. The ratio between the volume occupied by the protoplast (the structures bounded by the inner pericytoplasm membrane) and the volume occupied by the sporoplast (the structures bounded by the outer pericortex membrane) was calculated from measurements made on electron micrographs of medially thin-sectioned spores. Among the various spore types, an exponential increase in the heat resistance correlated directly with the wet density and inversely with the water content and with the protoplast/sporoplast volume ratio. Altogether the results supported a hypothesis that the extent of heat resistance is based in whole or in part on the extent of dehydration and diminution of the protoplast in the dormant spore, without implications about physiological mechanisms for attaining this state.

JOURNAL OF BACTERIOLOGY, May 1982, p. 643–648
0021-9193/82/050643-06$02.00/0

Vol. 150, No. 2

# Photometric Immersion Refractometry of Bacterial Spores†

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Received 3 June 1981/Accepted 22 October 1981

Photometric immersion refractometry was used to determine the average apparent refractive index ($\bar{n}$) of five types of dormant *Bacillus* spores representing a 600-fold range in moist-heat resistance determined as a $D_{100}$ value. The $\bar{n}$ of a spore type increased as the molecular size of various immersion solutes decreased. For comparison of the spore types, the $\bar{n}$ of the entire spore and of the isolated integument was determined by use of bovine serum albumin, which is excluded from permeating into them. The $\bar{n}$ of the sporoplast (the structures bounded by the outer pericortex membrane) was determined by use of glucose, which was shown to permeate into the spore only as deeply as the pericortex membrane. Among the various spore types, an exponential increase in the heat resistance correlated with the $\bar{n}$ of the entire spore and of the sporoplast, but not of the isolated perisporoplast integument. Correlation of the $\bar{n}$ with the solids content of the entire spore provided a method of experimentally obtaining the refractive index increment ($d\bar{n}/dc$), which was constant for the various spore types and enables the calculation of solids and water content from an $\bar{n}$. Altogether, the results showed that the total water content is distributed unequally within the dormant spore, with less water in the sporoplast than in the perisporoplast integument, and that the sporoplast becomes more refractile and therefore more dehydrated as the heat resistance becomes greater among the various spore types.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1982, p. 1307–1310 Vol. 43, No. 6
0099-2240/82/061307-04$02.00/0

# Wet and Dry Bacterial Spore Densities Determined by Buoyant Sedimentation†

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Received 24 August 1981/Accepted 14 March 1982

The wet densities of various types of dormant bacterial spores and reference particles were determined by centrifugal buoyant sedimentation in density gradient solutions of three commercial media of high chemical density. With Metrizamide or Renografin, the wet density values for the spores and permeable Sephadex beads were higher than those obtained by a reference direct mass method, and some spore populations were separated into several density bands. With Percoll, all of the wet density values were about the same as those obtained by the direct mass method, and only single density bands resulted. The differences were due to the partial permeation of Metrizamide and Renografin, but not Percoll, into the spores and the permeable Sephadex beads. Consequently, the wet density of the entire spore was accurately represented only by the values obtained with the Percoll gradient and the direct mass method. The dry densities of the spores and particles were determined by gravity buoyant sedimentation in a gradient of two organic solvents, one of high and the other of low chemical density. All of the dry density values obtained by this method were about the same as those obtained by the direct mass method.

JOURNAL OF BACTERIOLOGY, Jan. 1983, p. 436–442 Vol. 153, No. 1
0021-9193/83/010436-07$02.00/0


# Dielectric Characterization of Forespores Isolated from *Bacillus megaterium* ATCC 19213

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*Departments of Microbiology[1] and Electrical Engineering,[2] The University of Rochester, Rochester, New York 14642*

Received 21 June 1982/Accepted 12 October 1982

Isolated stage III forespores of *Bacillus megaterium* ATCC 19213 in aqueous suspensions were nearly as dehydrated as mature spores, as indicated by low dextran-impermeable volumes of ca. 3.0 ml per g (dry weight) of cells compared with values of ca. 2.6 for mature spores and 7.3 for vegetative cells. The forespores lacked dipicolinate, had only minimal levels of calcium, magnesium, manganese, potassium, and sodium, and were more heat sensitive than vegetative cells. The effective homogeneous conductivities and dielectric constants measured over a frequency range of 1 to 200 MHz indicated that the inherent conductivities of the forespores were unusually low, in keeping with their low mineral contents, but that the forespores could be invaded by environmental ions which could penetrate dielectrically effective membranes. Overall, our findings support the view that the dehydration of a forespore during stage III of sporogenesis may be the result of ion movements out of the forespore into the sporangium.

R.E. Marquis, E.L. Carstensen, G.R. Bender, S.Z. Child, in *Fundamental and Applied Aspects of Bacterial Spores*, G.J. Dring, D.J. Ellar, G.W. Gould, Ed. (Academic Press, London, 1983, in press).

## PHYSIOLOGICAL BIOPHYSICS OF SPORES

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Summary - Information obtained by use of simple, nondestructive, physical techniques is surveyed to obtain a clearer picture of the nature of dehydration during sporogenesis, the physical states of spore electrolytes and the relationship of heat resistance to mineralization. Major dehydration during stage III of sporogenesis seems to depend on osmotic-metabolic mechanisms involving the inverted, outer, forespore membrane. Subsequent dehydration and the maintenance of the dehydrated state appear to depend on cortical peptidoglycan elasticity. Minerals in the spore core are immobilized, but those in enveloping structures of many spores are mobile. Heat resistance appears to be acquired incrementally in association with dehydration and specific mineralization.

In R. Hakenbeck, J.-V. Höltje and H. Labischinski (ed.), The target of penicillin. de Gruyter and Co., Berlin. In press (1983).

## ELECTROCHEMICAL AND MECHANICAL INTERACTIONS IN BACILLUS SPORE AND VEGETATIVE MUREINS

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The condensed core or protoplast of a bacterial endospore is surrounded by a thick envelope of murein called the cortex. The elastic murein cortex is contained within an outer membrane and the complex of spore coats. The cortical murein is designed primarily for the mechanical function of maintaining the dehydrated state of the core, where the internal hydrostatic pressure has been estimated to be as much as 500 atmospheres or 50.65 MPascals. The cortex functions also in ion exchange and appears to be important for dormancy and for resistance to heat, pressure and other physical insults. Release of spores from dormancy during germination involves rapid and massive degradation of the cortical murein.

One of our main approaches to the physiological biophysics of cortical mureins involves non-destructive dielectric probing of living spores with current of 0.5 to 200 MHz frequency. This probing yields information on the electrochemical states of cortical mureins without causing any damage to the spores or upsetting in-situ molecular arrangements. We have also developed techniques for total ion exchange of viable spores to obtain various salt forms for dielectric studies. For comparisons, dielectric properties of isolated mureins and of vegetative cells have been measured.

We found that vegetative cell conductivities at low frequencies of about 1 MHz can be related directly to concentrations of mobile counterions, such as sodium or potassium ion, associated with charged groups in the murein complex. In essence, the murein complex of an intact cell or isolated cell wall behaves as a relatively open, highly hydrated, ion-exchange resin. Conductivity values at low ionic strength were approximately equal to those expected on the basis of need for only sufficient mobile counterions to balance the net negative charge of the network. In other words, the murein has sufficient molecular flexibility to allow for extensive intramolecular charge neutralization. Mobile counterions are required only for those charges which cannot be neutralized internally.

Because bacterial spores have a dielectrically effective membrane external to the murein layer, dielectric probing of the cortex required chemical removal of the coat-outer-membrane complex or the use of higher frequency current of about 50 MHz so that the outer membrane was capacitatively short circuited. We have studied two extreme cases in detail. The cortex of spores of Bacillus cereus strain terminalis had extremely low conductivity when the cells were in media of low ionic strength. Thus, the structure appeared to be nearly devoid of mobile counterions. In contrast, the cortex of spores of Bacillus megaterium ATCC 19213 had high inherent conductivity of about 0.13 mho/m, indicative of a sparsely cross-linked structure with an excess of charged groups that could not be neutralized by rearrangement of the murein matrix. The dielectric properties of these mureins will be interpreted in terms of their chemical structures and functional roles.

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